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Redox Abnormalities as a Vulnerability Phenotype for Autism and Related Alterations in CNS Development

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13. SUPPLEMENTARY NOTES

14. ABSTRACT:

We hypothesize that low systemic redox potential (GSH/GSSG; cysteine/cystine) reflects a vulnerability phenotype that is associated with regressive autism and is predictive of the risk of developing autism. The redox vulnerability phenotype is associated with epigenetic alterations in primary immune cells that may be reversible with restoration of intracellular redox potential. The hypothesis predicts that children with regressive autism and high risk (developmentally-delayed) children who are subsequently diagnosed with autism will exhibit lower redox potential compared to age-matched unaffected control children. It also predicts that low redox potential from these children will be associated with epigenetic modifications in DNA methylation and histone acetylation/methylation that are reversible with treatment to restore redox potential. In Aim 1 we will determine whether redox potential in immune cells can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism. We will also evaluate immune redox potential from high risk developmentally delayed children to determine whether redox status is predictive of subsequent development of autism. In Aim 2, we will determine whether immune cells from autistic children are associated with altered cytokine patterns, macrophage/T cell DNA methylation, and chromatin histone methylation compared to control children.

15. SUBJECT TERMS

None provided.

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PROGRESS REPORT 2009

2008-2009 Accomplishments

Project 1 PI: S. Jill James PhD

INTRODUCTION:

Based on our preliminary studies, we hypothesize that children with autism spectrum disorders (ASD) have a more oxidized metabolic status than normal children. The goal of Aim 1 of this project is to better define the functional implications of redox abnormalities associated with autism and to study the predictive potential of the GSH/GSSG redox ratio as a biomarker for autism. The goal of Aim 2 is to determine whether targeted treatment to increase redox potential will restore cytokine balance and reverse epigenetic alterations in primary immune cells from children with ASD. A summary of our progress to date is summarized below in the body of the report following the format of Project 1 SOW and is accompanied by preliminary data year with interpretation of these early results.

BODY:

***Aim 1*: Determine whether redox potential in primary immune cells can be used as a biomarker for regressive autism and whether it is predictive of the subsequent diagnosis of autism.**

a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing NIH grant (1RO1HD051873) (months 1-4) **Done**

b) Selection of children with 50 regressive autism, 50 infantile autism and 50 age-matched control boys; Selection of 50 children with developmental delay (DD) with diagnosis of autism; 50 children with DD without autism; 50 age-matched control children (months 1-2)

We have identified 30 children with sudden onset regression, 20 children with infantile autism, and 50 control children. Figure 1 and 2 present our data to date comparing GSH/GSSG redox ratio and GSSG levels between regressive onset, infantile onset and age matched controls.

Interpretation: Trend toward lower GSH/GSSG and higher oxidized GSSG levels in children with regressive compared to early onset autism.

Figure 1

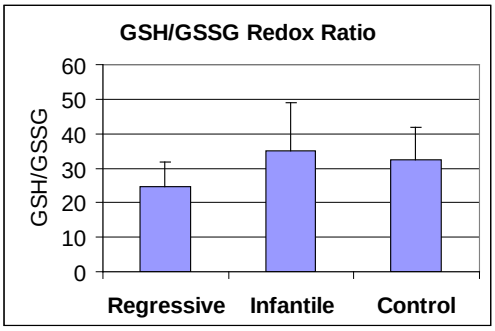
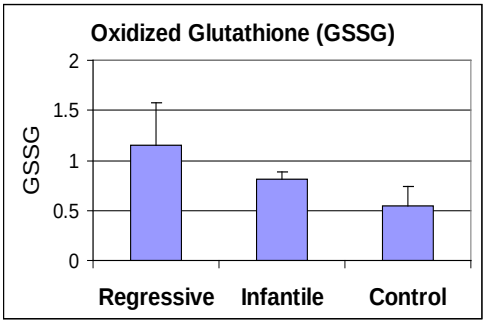


Figure 2



c) Purification of leukocytes from 300 frozen RBC pellets previously centrifuged and stored. Preparation of extract and protein content. (ongoing; years 1-3)

We will use extracts from plasma and primary cells to identify redox-related predictive biomarkers of regressive autism in newly recruited cases and controls. Because

extracts from frozen RBC pellets proved to be unstable and not reproducible, we are collecting data on fresh primary leukocytes from newly recruited patients. Preliminary results are presented below comparing plasma GSH/GSSG in children who failed the MCHAT (high risk) and those that passed the M-CHAT (low risk).

Table 1:

	Total GSH (µmol/L)	Free GSH (µmol/L)	GSSG (µmol/L)	fGSH/GSSG redox ratio
M-CHAT Fail (n-15)	6.0 ± 1.1	1.8 ± 0.4	0.25 ± 0.2	8.2 ± 3.4
M-Chat Pass (n=7)	6.2 ± 1.6	2.2 ± 0.4	0.14 ± 0.1	21.3 ± 13.3
p value	ns	0.02	<0.001	0.001

<u>Interpretation</u>: These preliminary results are consistent with a lower GSH/GSSG redox potential in children who are at high risk of developing autism. These results may serve as predictive biomarkers if they are maintained with higher number of samples.

e) HLPC-ESI-MS analysis of 300 blood samples (300 x 3 runs each = 900) and mouse tissue analysis for GSH/GSSG and cysteine/cystine. Ongoing; years 1.5-3).
The preliminary results for the GSH/GSSG and Cysteine/Cystine redox ratio in 55 blood samples from autistic children and 51 age-matched control children are presented Figure 3 and 4 below.
<u>Interpretation</u>: Both GSH/GSSG (reflection of intracellular redox status) and Cysteine/Cystine (reflection of extracellular redox status) were significantly lower in autistic compared to control children.

Figure 3: GSH/GSSG ratio

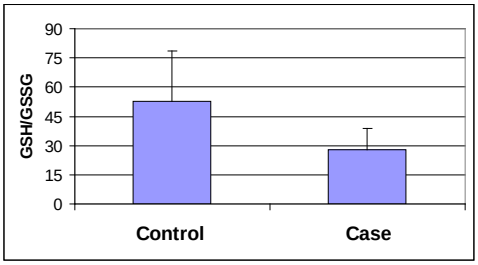
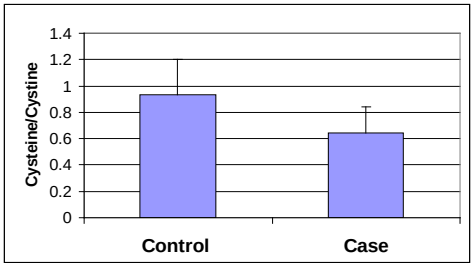


Figure 4: Cysteine/Cystine ratio



f) Statistical Analysis and manuscript writing: (Year 3)

Deliverables: We anticipate 2 publications in major peer-reviewed journals. The discovery of a more oxidized phenotype among children with regressive autism and/or as a biomarker for the risk for developing autism would provide new insights into the etiology of autism as well as earlier detection and new treatment strategies

<u>Aim 2</u>: Determine whether targeted treatment to increase glutathione redox potential in autistic children will restore cytokine balance and reverse epigenetic alterations in primary immune cells.

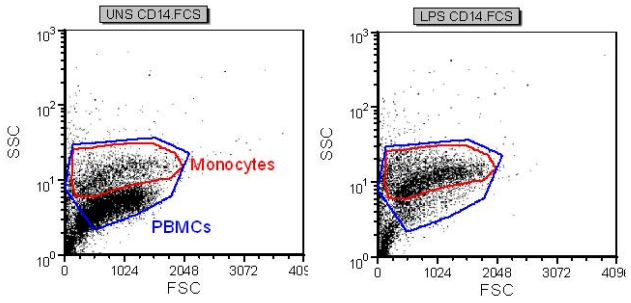
a) DoD regulatory review and approval of our UAMS IRB-approved protocol and consents for our ongoing Arkansas Children's Hospital Foundation intervention study (months 1-4) **Done**

b) Sample collection before and after nutritional intervention to increase GSH/GSSG redox in 30 autistic (total 60 samples) and 30 controls in our IRB-approved clinical trial (Ongoing; yrs 1-3)
We have recruited seven autistic children into our double-blind placebo controlled study to date.

c) Purification of monocyte/macrophages and T cells from 90 fresh blood samples (years 1-3)
Methodology for macrophage and T cell isolation has been successfully accomplished. We are able to obtain 75% pure monocytes and 90% pure T cells
<u>Interpretation</u>: We are able to define macrophage and T cell subsets using monoclonal antibodies and flow cytometry.

Figure 5: Monocyte Purification

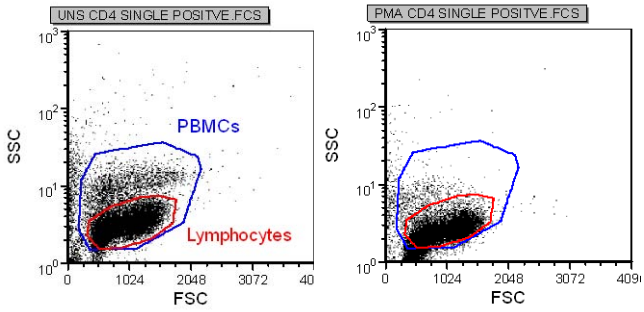
Monocytes:



Unstimulated and LPS stimulated and isolated monocytes light scatter plots to show elimination of the lymphocyte population

Figure 6: T cell Purification

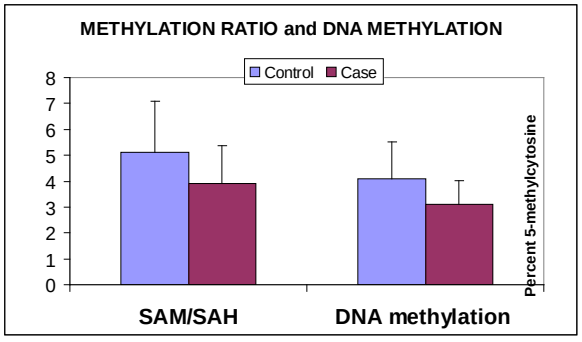
T cells:



Unstimulated and PMA/iono stimulated and isolated T-cells light scatter plots to show elimination of the monocyte population

d) Leukocyte global DNA methylation determination using HLPC-ESI-MS technology before and after targeted intervention to increase GSH (n=60 + 30 control samples; ongoing; years 1-3)
We have completed DNA extraction and DNA methylation on ~100 cases and controls – recruitment is on-going. Preliminary results are presented below.

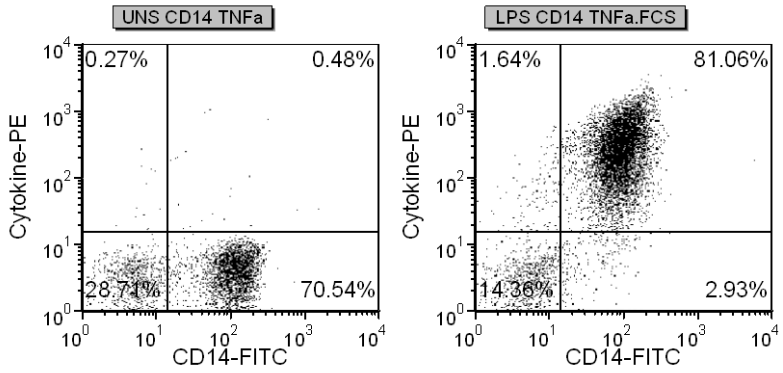
Figure 7: Global DNA methylation levels and SAM/SAH ratios in case and control children



<u>Interpretation</u>: Preliminary results suggest that global DNA methylation is significantly decreased in autistic compared to control children and is associated with lower methylation potential (SAM/SAH)

e) Determine intracellular cytokine patterns with flow cytometry in stimulated leukocytes from 30 controls and 30 autistic children before and after intervention to increase GSH (yrs 1-3)

Figure 8: Intracellular TNF-α cytokine production in unstimulated and LPS stimulated monocytes



Unstimulated LPS stimulated

f) Determine gene-specific histone acetylation/methylation patterns in the IL-4 and IFNγ genes in T cells using chromatin immunoprecipitation before and after intervention (n=90; years 1-3)

We have worked up methodology of native ChIP and quantification of histone H3K9 in the promoter region of the TNF-α gene which has a CpG island.

Figure 9: Histone H3K9 methylation

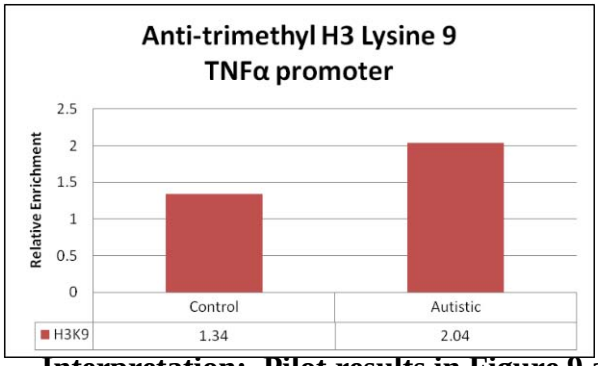
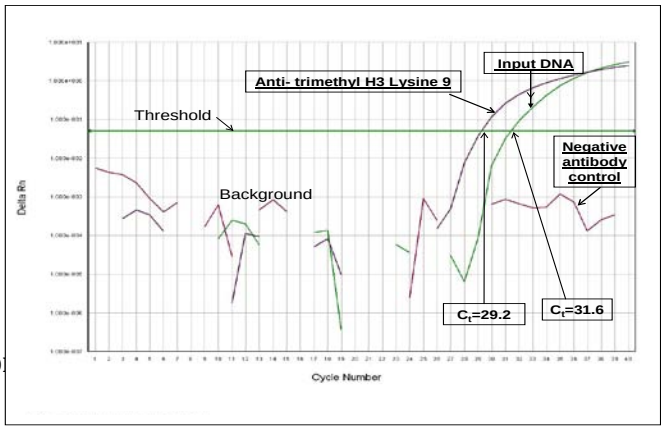


Figure 10: Raw data



<u>Interpretation</u>: Pilot results in Figure 9 are co
region histone methylation

g) Statistical Analysis and manuscript writing: (Year 3)

Deliverables: We anticipate 2-3 publications in major peer-reviewed journals. We expect that targeted nutritional intervention previously shown to increase plasma GSH/GSSG will increase leukocyte GSH/GSSG and will also restore cytokine balance/epigenetic dysfunction to improve immune function in autistic children. We anticipate that this project will be the first to provide definitive evidence for epigenetic dysregulation of immune function in autism.

Problems encountered and solutions:

1. **We were unable to get reproducible results using our stored frozen cell pellets as originally described in the protocol. Instead, we will only be able to measure intracellular GSH/GSSG on fresh primary cells from newly recruited children. Recruitment is ongoing and we anticipate we will have sufficient number of subjects for statistically meaningful results.**
2. **We have found that the volume of blood obtainable varies between children. Some children are more difficult to stick and we may only get 3 ml instead of 20ml needed to do full analysis. We have established a priority of assays depending of final volume obtained: PBMCs > monocyte > T cells.**
3. **We have found that histone purification from primary cells is also dependent on cell volume obtained and has not been reproducible. We have successfully purified histones from lymphoblastoid cell lines obtained from children with autism and unaffected controls. We will submit a request to revise the protocol to do assays on cell lines rather than primary cells to assure reproducibility and meaningful results.**

KEY RESEARCH ACCOMPLISHMENTS

- GSH/GSSG redox ratio and GSSG level analysis was completed on 30 sudden-onset, 20 infantile, and 50 control samples and is expected to continue until 50 samples from each group are collected.
- Preliminary results from 15 high-risk and 7 low-risk children indicate a lower GSH/GSSG redox ratio in high-risk children who failed the MCHAT.
- Preliminary results show a lower intracellular redox ratio in primary lymphocytes from autistic children compared to controls.
- Global DNA methylation also appears to be lower in autistic children.
- Method development for flow cytometry intracellular cytokine production is complete
- Chromatin Immunoprecipitation method development for TNF-a promoter region histone methylation completed in autism and control cell lines.

REPORTABLE OUTCOMES

1. Approval from the Autism Tissue Program to obtain frozen brain tissues form autistic and control individuals.
2. NIH R01 ARRA Stimulus Grant application (not funded). Our data showing histone mehtylation in the TNF-a promoter was used as preliminary evidence to study similar modifications in frozen brain tissue from autistic and control individuals.
3. Private donation received to obtain preliminary evidence for resubmission of frozen brain histone methylation study.

CONCLUSION

Definitive conclusions not possible in the first year of the grant with data collection on-going.

REFERENCES

None

APPENDICES

None